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Oligonucleotide Analogues Containing a C3'–NH–C(O)–CH_2–C5' Amide Internucleotide Bond

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A dinucleotide containing a C3'–NH–C(O)–CH_2–C5' amide internucleotide bond was synthesized by the interaction of 3'-deoxy-3'-amino-5'-*O*-(*tert*-butyldimethylsilyl)thymidine with 3'-*O*-benzyl-2'-*O*-*tert*-butyldimethylsilyl-5'-deoxy-5'-carboxymethylribosylthymine, which was obtained from 2'-*O*-acetyl-3'-*O*-benzyl-5'-deoxy-5'-ethoxycarbonylmethylribosylthymine through the methanolysis of the acetyl group followed by silylation of liberated hydroxyl and ester saponification. After standard manipulation with protecting groups, the dinucleotide was converted into 3'-*O*(2-cyanoethyl-*N,N*-diisopropylphosphoramidite), which was used for the synthesis of modified oligonucleotides on an automated synthesizer. The melting curves of the duplexes formed by modified and complementary natural oligonucleotides were registered, and the melting temperatures and thermodynamic parameters of the duplex formation were calculated. The introduction of a single modified bond into the oligonucleotide led to an insignificant decrease in the melting temperature of these duplexes as compared to unmodified ones.

Key words: oligonucleotides, analogues, amide internucleotide bond, hybridization, thermal stability

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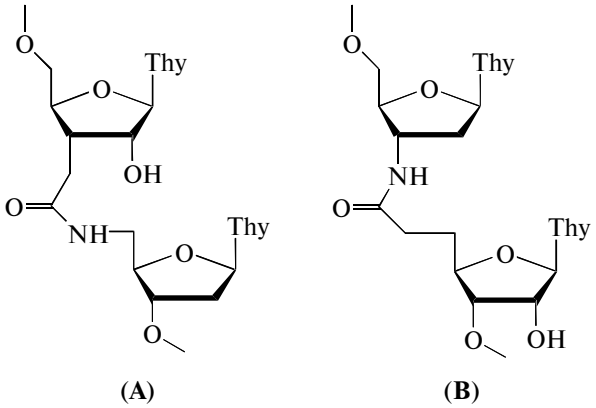
INTRODUCTION

Therapy with synthetic oligonucleotide analogues (antisense therapy) is one of the most prospective methods for the treatment of viral and oncological diseases [1–3].[2] The concept of the method is the possibility of the inhibition of the translation of a target gene through the specific binding of a synthetic oligonucleotide to the corresponding region of mRNA. An important criterion for choosing antisense oligonucleotides is the stability of their duplexes with complementary natural deoxyribo- or ribooligonucleotides [4]. The melting temperature of the modified duplexes is usually used as a quantitative measure of their stability. This temperature should be comparable with or higher than that of the corresponding natural duplexes.

Over the last 15 years, a vast majority of oligonucleotide analogues have been synthesized with modified heterocyclic bases, carbohydrate residues, or internucleotide bonds (see reviews [5–7]). Few of them, however, meet the stability criterion. The most prospective are oligonucleotides in which there are a phosphate internucleotide bond replaced by a phosphorothioate one [8, 9], 2'-*O*-alkyloligonucleotides [10], peptide nucleic acids (PNAs) [11, 12], oligonucleotides containing morpholino cycle instead of furanose [13, 14], bicyclic sugar (LNA) [15, 16], and an amide internucleotide bond [17, 18].

In our previous paper [19], we described the synthesis and properties of oligonucleotides containing the C3'–CH_2–C(O)–NH–C5' amide internucleotide bond (structure (**A**) in Scheme 1). The present work is devoted to the synthesis and the study of the physicochemical and hybridization properties of oligonucleotide analogues containing a reverse sequence of atoms in the internucleotide bond (structure (**B**) in Scheme 1).



Scheme 1. Dinucleotides containing an internucleotide amide bond.

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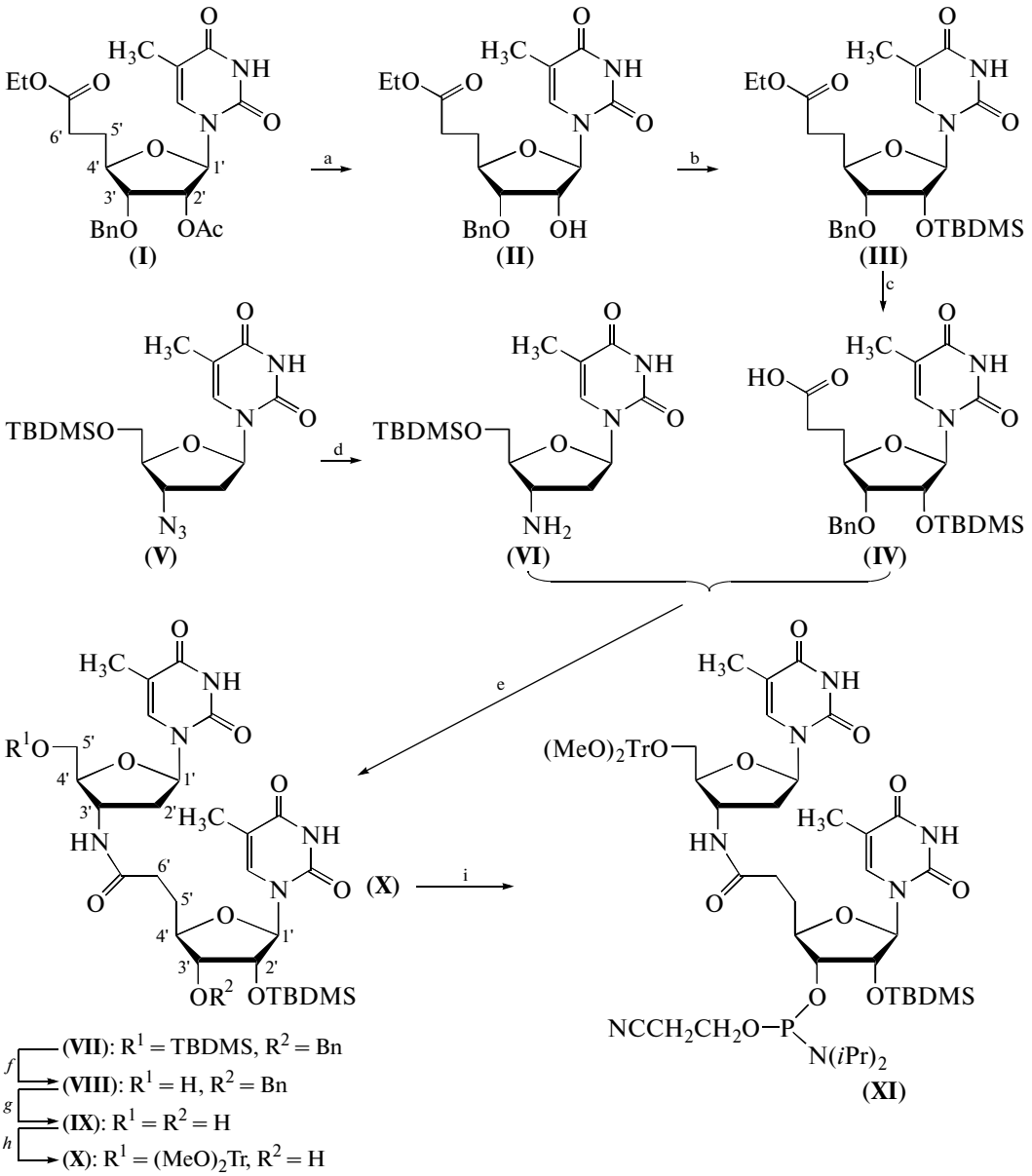
[2] Abbreviations: Bn, benzyl; DIPEA, diisopropylethylamine; TBDMS-Cl, *tert*-butyldimethylsilyl chloride; TEAA, triethylammonium acetate; $(MeO)_2$Tr, dimethoxytrityl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TBAF, tetrabutylammonium fluoride; cT, 5'-deoxy-5'-carboxymethyl-5'-ribosylthymine; Ta, 3'-amino-3'-deoxythymidine.

RESULTS AND DISCUSSION

3'-Amidite dinucleotide (**B**) for the synthesis of oligonucleotide analogues was prepared according to Scheme 2. Previously prepared 2'-*O*-acetyl-3'-*O*-benzyl-5'-deoxy-5'-ethoxycarbonylmethylribosylthymine (**I**) [20] was subjected to methanolysis, which resulted in a 93% yield of nucleotide (**II**) containing a deprotected 2'-hydroxy group. Compound (**II**) was converted into a 2'-*O*-TBDMS derivative (**III**) by the interaction with *tert*-butyldimethylsilyl chloride, which led to free acid (**IV**) in a yield of 91% after alkaline hydrolysis and the subsequent acidification.



Scheme 2. (a) K_2CO_3, MeOH; (b) TBDMS-Cl, imidazole, acetonitrile; (c) KOH, ethanol/water; (d) $HCOONH_4$, Pd/C, MeOH; (e) carbonyldiimidazole, CH_2Cl_2; (f) 90% TFA; (g) H_2, Pd/C, THF; (h) $(MeO)_2TrCl$, pyridine; and (i) $NCCH_2CH_2OP(NiPr_2)Cl$, DIPEA, CH_2Cl_2.

3'-Amino-3'-deoxy-5'-*O*-TBDMS-thymidine (**VI**) was synthesized by the reduction of the corresponding azide (**V**) with ammonium formate in the presence of 10% Pd/C.

Completely protected dinucleotide (**VII**) was prepared in a yield of 81% by the coupling of acid (**IV**) with amine (**VI**) in the presence of carbonyldiimidazole. The primary TBDMS protecting group was selectively removed with trifluoroacetic acid at 0°C. An attempt to remove the benzyl group in dinucleotide (**VIII**) by hydrogenation in ethanol over 20% $Pd(OH)_2$ led to a mixture of 3'- and 2'-*O*-TBDMS isomers in a

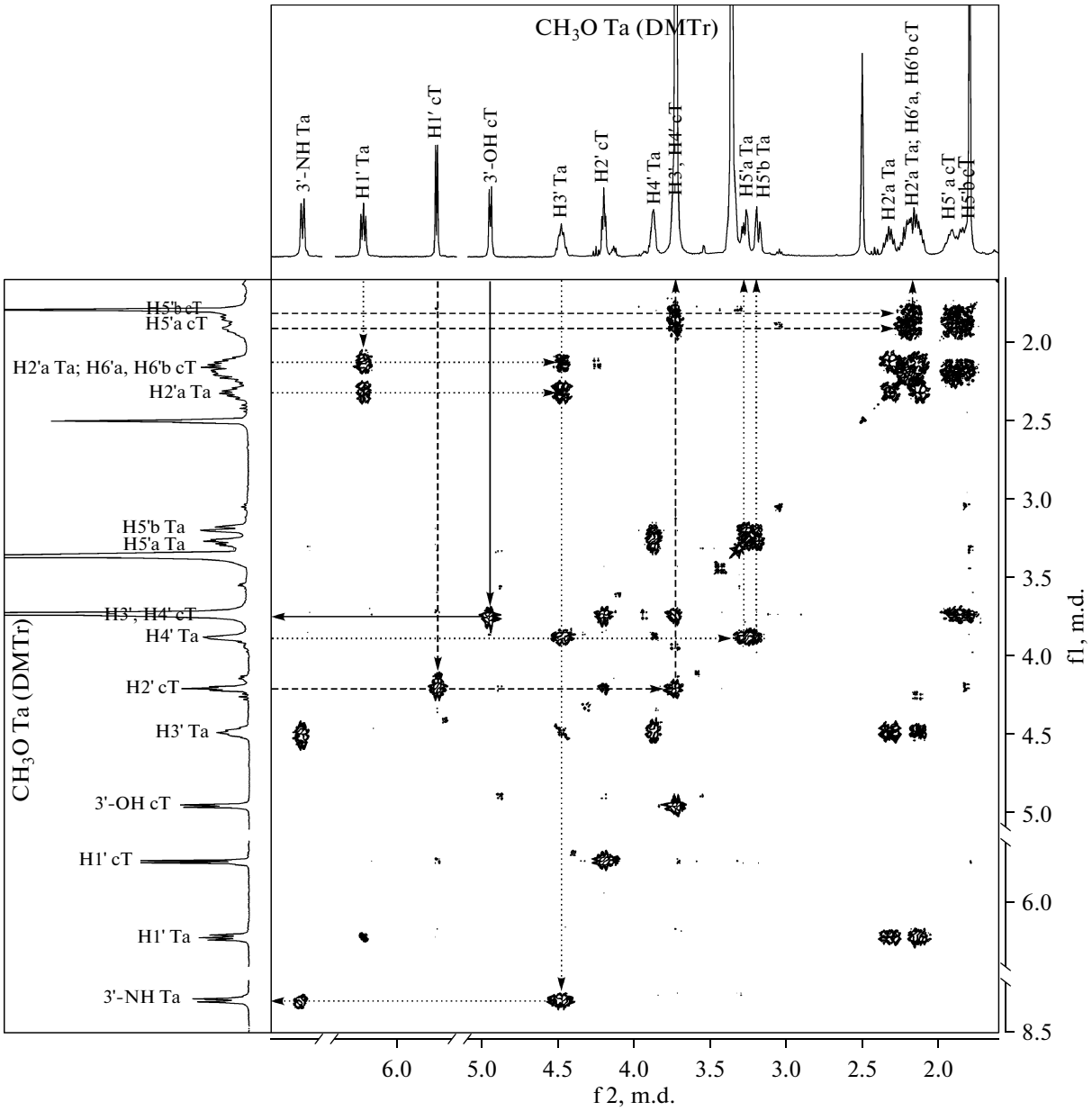


Fig. 1. Fragment of the COSY DQF spectrum showing the correlation between protons in the carbohydrate cycles of dinucleotide (**X**). cT is the 5'-deoxy-5'-carboxinethylribosylthymine residue and Ta is the 3'-amino-3'-deoxythymidine residue. The attribution of signals to cT protons is designated by the dotted line, to Ta protons, by the points.

1 : 0.7 ratio according to the NMR spectrum. It was shown [21] that the migration of the TBDMS group is reduced in dry aprotic solvents. Indeed, while hydrogenating in absolute tetrahydrofuran over 10% Pd/C for 5 h, only a 2'-*O*-TBDMS derivative (**IX**) was obtained. The subsequent dimethoxytritylation led to dinucleotide (**X**), the interaction of which with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite in the presence of diisopropylethylamine resulted in phosphoroamidite (**XI**).

The structure of all of the synthesized compounds was confirmed by the ^{1}H NMR spectra. In the case of dinucleotides (**VII**)–(**X**), the attribution of signals to the nucleotide protons was performed by COSY DQF spectra. As an example, the two-dimensional spectrum of dinucleotide (**X**) is presented in Fig. 1.

This spectrum allows for the unambiguous identification of the proton signals in both nucleotide residues. Since the spectrum was registered in DMSO-d_6, it revealed the signals of exchanging protons, namely, hydroxyl groups, which were identified by disappearance while adding D_2O to the sample. The proton of the secondary hydroxyl group (doublet) gives a cross peak with the 3'-proton of the cT residue (solid line in

Table 1. Synthesized oligonucleotides and their mass spectra (**B** is modified dinucleotide, see Scheme 1)

Code	Sequence, 5' ⟶ 3'	Mass spectra	
		Obtained, m/z	Calculated for $[M-H]^-$
Modified oligonucleotides			
M1	G-**B**-TT-TT-TT-TT-G	3614.9	3614.45
M2	G-TT-TT-TT-TT-**B**-G	3614.7	3614.45
M3	G-TT-TT-**B**-TT-TT-G	3614.2	3614.45
M4	G-**B**-TT-**B**-TT-**B**-G	3568.4	3568.59
Natural oligonucleotides			
S1	G-TT-TT-TT-TT-TT-G	3637.8	6337.38
S2	C-AA-AA-AA-AA-AA-C	3646.9	3647.46

Table 2. Thermodynamic parameters of duplex formation from single strands

Duplex	$c \times 10^6$, M	$\Delta H°$, kcal/mol	$\Delta S°$, cal mol^{-1} K^{-1}	T_m, °C ± 0.5	T_m, °C normalized*	ΔT_m, °C**
S1·S2	10.37	−90.2 ± 0.7	−266.1 ± 2.3	36.2	36.2	
M1·S2	4.86	−88.1 ± 1.7	−260.6 ± 5.5	33.7	35.4	−0.8
M2·S2	6.79	−89.2 ± 1.0	−263.2 ± 3.2	34.9	35.7	−0.5
M3·S2	10.18	−91.4 ± 1.5	−271.5 ± 4.7	34.5	34.5	−1.7
M4·S2	10.30	−86.4 ± 1.2	−258.7 ± 3.9	31.1	31.0	−5.2

Notes: * Calculated for a 10^{-5} M concentration.
** Difference between T_m of modified and natural duplexes.

Fig. 1), which unambiguously points to the presence of a free 3'-hydroxyl, i.e., 2'-*O*-TBDMS derivative. Moreover, the 2D spectrum allows for the identification of overlapping signals. The signals of H3' and H4' in the cT residue in the 1D spectrum are overlapped and fully masked by the signal of the CH_3 groups in the dimethoxytrityl group of another nucleotide. In the 2D spectrum, the cross peaks with H2' and with H5'a and H5'b in the cT residue allow for the identification of the H3' and H4' signals, respectively.

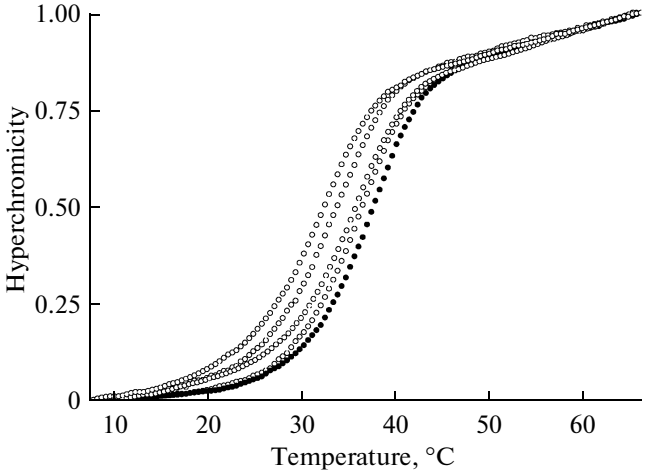


Fig. 2. Experimental melting curves for synthesized duplexes (left to right): **M4·S2**, **M1·S2**, **M3·S2**, **M2·S2**, **S1·S2**. For convenience, hyperchromicity was normalized to 1.

All of the dinucleotides were characterized by MALDI mass spectra (see the Experimental section).

Phosphoramidite (**XI**) was used for the synthesis of oligonucleotide analogues. In addition, complementary natural oligonucleotides were prepared (Table 1).

The structure of the synthesized oligonucleotides was confirmed by mass spectra (Table 1). The thermal dissociation profiles of the natural and modified duplexes were registered (Fig. 2).

The obtained curves allowed for the calculation of the thermodynamic parameters of the duplex formation from single strands using the two-state model (Table 2). The temperature dependence of the adsorption of a duplex (A_d) and single strands (A_{ss}) was taken into account. Since the melting temperature of short duplexes depends on the concentration, the experimental melting temperatures were normalized to the standard 10^{-5} M concentration of the strands (second to last column in Table 2).

As seen from Table 2, a single modification caused only an insignificant decrease in the duplex melting

temperature as compared to that of the corresponding natural complex.

Thus, the prepared oligonucleotides meet the stability criterion applicable to antisense oligonucleotides.

EXPERIMENTAL

We used pyridine, chloroform, methylene chloride, dimethylformamide, ethyl acetate, acetonitrile, tetrahydrofuran (purity grade, Khimmed, Russia), abs. methanol (Panreac, Spain), ammonium formiate, triethylamine, acetanhydride (Fluka, Switzerland), dimethoxytrityl chloride, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, and DEAE-Toyopearl 650M (Aldrich, United States). 3'-Deoxy-3'-azidothymidine was kindly provided by the AZT Association (Russia). Dimethylformamide, methylene chloride, and acetonitrile were dried by reflux over phosphorus pentoxide; pyridine, over calcium hydride; and tetrahydrofuran, over lithium alumohydride.

The NMR spectra (δ, ppm; SSCC, Hz) of compounds in DMSO-d_6 (if not specially mentioned) were registered on a Bruker AMXIII-400 spectrometer with a working frequency of 400 MHz. Spectra were processed by the MestReNova 5.3.0 program, (Mestrelab Research SL).

The duplexes' melting curves were recorded on a UV 160-A spectrophotometer (Shimadzu, Japan) supplied with a thermostatic device. The absorption of a duplex (A_{260}) was registered in 0.5°C intervals. The calculation of the thermodynamic parameters was performed by the nonlinear regression method in the DataFit 9.0.059 program (Oakdale Engineering, United States).

MALDI analysis of modified and natural oligonucleotides was carried out on an MS-30 mass spectrometer (Kratos, Japan) in the linear mode using negative-ion registration.

Oligonucleotides were synthesized on an automated 3400 DNA Synthesizer (Applied Biosystems, United States). The introduction of natural nucleotides was carried out according to the standard protocol; when a modified dinucleotide was used, the coupling time was increased by a factor of three. The silyl protecting group was removed by 1 M TBAF in THF followed by desalting on a column with 0.5 ml of DEAE Toyopearl using 0.1 M TEAA, pH 7, as an eluent. The oligonucleotide was eluted by 1 M TEAA. The purification of oligonucleotides was performed by reverse-phase HPLC on Hipersil C18 columns (25 cm × 4 mm^2) in a 0.05 M TEAA buffer. $(MeO)_2$Tr-Protected oligomers were isolated in an acetonitrile gradient (10–50%). Fully deprotected oligonucleotides were additionally purified in a gradient from 0 to 25% in the same buffer.

For the preparative isolation, we used flash chromatography on Kieselgel 60, 40–63 μm (Merck, Germany). TLC was fulfilled on Kieselgel 60 F_{254} plates (Merck, Germany) in systems of ethanol–methylene chloride in ratios of 1 : 30 (A), 1 : 19 (B), 1 : 9 (C), and 1 : 9 + 0.1% TEA (D).

3'-*O*-Benzyl-5'-deoxy-5'-ethoxycarbonylmethylribosylthymine (II). K_2CO_3 (0.17 g, 1.2 mmol) was added to the solution of 2'-*O*-acetyl-3'-*O*-benzyl-5'-deoxy-5'-ethoxycarbonylmethyl-5-ribosylthymine (**I**) [20] (1.4 g, 3.0 mmol) in abs. methanol (50 ml), and the mixture was stirred for 4 h. Acetic acid (1.5 ml) was then added with stirring for 5 min, and the solvent was evaporated. The residue was divided between water (50 ml) and chloroform (50 ml). The water layer was extracted with chloroform (2 × 20 ml). The combined chloroform extracts were washed with a saturated $NaHCO_3$ solution (3 × 50 ml) and water, dried with anhydrous Na_2SO_4, and evaporated. The residue was separated by chromatography on a silica-gel column using a methylene chloride–2% methanol mixture as an eluent. Fractions containing the product were combined and evaporated. Product (**II**) was obtained in a solid foam form in a yield of 1.18 g (93%), R_f 0.27 (A). ^{1}H NMR ($CDCl_3$): 8.774 (1 H, bs, H3), 7.29–7.39 (5H, m, Ph), 7.047 (1 H, bd, *J* 1.14, H6), 5.601 (1 H, d, $J_{1',2'}$ 4.23, H1'), 4.639 (2 H bs, PhC$\underline{H}_2$O), 4.256 (1 H, dd, $J_{1',2'}$ 4.23, $J_{2',3'}$ 5.77, H2'), 4.111 (2 H, qv, *J* 7.14, OC$\underline{H}_2$CH$_3$), 4.012 (1 H, m, H4'), 3.144 (1 H, dd, $J_{2',3'}$ 5.77, $J_{3',4'}$ 3.75, H3'), 2.434 (1 H, m, H6'a), 2.367 (1 H, dd, $J_{5',6'b}$ 7.22, $^2J_{6'a,6'b}$ 16.59, H6'b), 2.011 (1 H, m, H5'a), 1.927 (1 H, m, H 5'b), 1.913 (3 H, d, *J* 1.14, 5-CH$_3$), 1.232 (3 H, t, *J* 7.14, OCH$_2$C$\underline{H}_3$).

3'-*O*-Benzyl-2'-*O*-*tert*-butyldimethylsilyl-5'-deoxy-5'-ethoxycarbonylmethylribosylthymine (III). A mixture of nucleotide (**II**) (1.08 g, 2.58 mmol) and imidazole (0.7 g, 10.3 mmol) was evaporated with abs. acetonitrile (3 × 5 ml) and dissolved in abs. acetonitrile (5 ml). After the addition of TBDMS-Cl (0.97 g, 6.45 mmol), the reaction mixture was kept overnight under stirring. Water (2 ml) was then added, and the mixture was stirred additionally for 30 min followed by evaporation. The residue was dissolved in CH_2Cl_2 (50 ml), washed with 10% $KHSO_4$ (2 × 20 ml) and then with saturated $NaHCO_3$ (2 × 20 ml), dried with anhydrous Na_2SO_4, and evaporated. The residue was chromatographed on a silica-gel column (3.5 × 20 cm) with a methylene chloride–1% methanol mixture. Fractions containing the product were combined and evaporated. Product (**III**) was obtained in a solid foam form in a yield of 1.33 g (97%), R_f 0.68 (A). ^{1}H NMR ($CDCl_3$): 8.396 (1 H, bs, H3), 7.26–7.37 (5 H, m, Ph), 7.126 (1 H, bd, *J* 1.11, H6), 5.677 (1 H, d, $J_{1',2'}$ 3.53, H1'), 4.704 (1H, d, $^2J_{a,b}$ 11.74, Ha PhC$\underline{H}_2$O), 4.470 (1 H, d, $^2J_{a,b}$ 11.74, Hb PhC$\underline{H}_2$O), 4.378 (1 H, t, *J* 4.30, H2'), 4.129 (1 H, m, H4'), 4.108 (2 H, qv, *J* 7.14, OC$\underline{H}_2$CH$_3$), 3.559 (1 H, dd, $J_{2',3'}$ 5.11, $J_{3',4'}$ 5.75,H3'), 2.450 (2 H, m, H6'a, H6'b), 2.036 (1 H, m, H5'a), 1.949 (1 H, m, H5'b), 1.936 (3 H, d, *J* 1.11, 5-CH$_3$), 1.226 (3 H, t, *J* 7.14, OCH$_2$C$\underline{H}_3$), 0.885 (9 H, s,

$(CH_3)_3CSi$), 0.088 (3 H, s, CH_3Si), 0.083 (3 H, s, CH_3Si).

3'-*O*-Benzyl-2'-*O*-*tert*-butyldimethylsilyl-5'-deoxy-5'-carboxymethylribosylthymine (IV). A solution of 4 N KOH (1.6 ml) was added to compound (**III**) (1.3 mg, 2.4 mmol) in ethanol (4.2 ml). After stirring the mixture for 1 h, water (20 ml) and 1 M $KHSO_4$ (7 ml, pH 2) were added followed by extraction with CH_2Cl_2 (4 × 20 ml). The extracts were washed with water, dried with anhydrous Na_2SO_4, and evaporated. Acid (**IV**) was obtained in a solid foam form in a yield of 1.1 g (93%), R_f 0.33 (D). ^{1}H NMR ($CDCl_3$): 9.311 (1 H, bs, H3), 7.26–7.39 (5 H, m, Ph), 7.148 (1 H, bd, *J* 1.16, H6), 5.631 (1 H, d, $J_{1',2'}$ 3.13, H1'), 4.699 (1 H, d, $^2J_{a,b}$ 11.69, Ha PhC$\underline{H}_2$O), 4.455 (1 H, d, $^2J_{a,b}$ 11.69, Hb PhC$\underline{H}_2$O), 4.410 (1 H, t, *J* 3.85, H2'), 4.153 (1 H, m, H4'), 3.557 (1 H, dd, $J_{2',3'}$ 5.01, $J_{3',4'}$ 5.99, H3'), 2.507 (2 H, m, H6'a, H6'b), 2.045 (1 H, m, H5'a), 1.961 (1 H, dd, $J_{4',5'b}$ 9.11, $^2J_{5'a,5'b}$ 16.44 H5'b), 1.961 (1 H, dd, H5'b) 1.908 (3 H, d, *J* 1.16, 5-CH_3), 0.887 (9 H, s, $(CH_3)_3CSi$), 0.097 (3 H, s, CH_3Si), 0.087 (3 H, s, CH_3Si).

3'-Azido-3'-deoxy-5'-*O*-(*tert*-butyldimethylsilyl)thymidine (V). A mixture of 3'-deoxy-3'-azidothymidine (1.07 g, 4 mmol) and imidazole (90.41 g, 6 mmol) was evaporated with abs. acetonitrile (3 × 5 ml) and dissolved in abs. acetonitrile (10 ml) followed by the addition of TBDMS-Cl (0.75 g, 5 mmol) under stirring. The mixture was kept overnight at room temperature. After the addition of water (10 ml), the mixture was evaporated and divided between water (50 ml) and ethyl acetate (50 ml). The water layer was extracted with ethyl acetate (20 ml); the combined organic layers were washed with 10% aqueous $KHSO_4$ (2 × 20 ml), saturated aqueous $NaHCO_3$ (2 × 20 ml), and water; dried with anhydrous Na_2SO_4; and evaporated. Azide (**V**) was obtained in crystal form in a yield of 1.4 g (92%), R_f 0.64 (B), t_m 89–90°C (from an ethyl acetate–hexane mixture, 2 : 1). ^{1}H NMR ($CDCl_3$): 8.383 (1 H, bs, H3), 7.412 (1 H, bd, *J* 1.06, H6), 6.202 (1 H, t, *J* 6.52, H1'), 4.226 (1 H, m, H4'), 3.960 (1 H, m, H3'), 3.935 (1 H, dd, $J_{4',5'a}$ 2.62, $^2J_{5'a,5'b}$ 11.12, H5'a), 3.796 (1 H, dd, $J_{4',5'b}$ 2.02, $^2J_{5'a,5'b}$ 11.12, H5'b) 2.423 (1 H, m, H2'a), 2.217 (1 H, m, H2'b), 1.912 (3 H, d, *J* 1.06, 5-CH_3), 0.930 (9 H, s, $(CH_3)_3CSi$), 0.123 (6 H, s, $(CH_3)_2Si$).

3'-Amino-3'-deoxy-5'-*O*-(*tert*-butyldimethylsilyl)thymidine (VI). 10% Pd/C (0.11 g) and then ammonium formate (1.14 g) were added to a solution of azide (**V**) (1.14 g, 3 mmol) in abs. methanol (24 ml). The mixture was boiled under stirring for 2 h. After cooling, the mixture was filtered through celite, washed with methanol (2 × 15 ml), and evaporated. The residue was mixed with saturated $NaHCO_3$ (50 ml) and extracted with CH_2Cl_2 (4 × 20 ml). The combined extracts were washed with water (20 ml), dried with anhydrous Na_2SO_4, and evaporated. Amine (**VI**) was obtained in crystal form in a yield of 94%, R_f 0.28 (b), T_m 89–90°C (from ethyl acetate). ^{1}H NMR ($CDCl_3$): 8.193 (1 H, bs, H3), 7.475 (1 H, bd, *J* 1.18, H6), 6.238 (1 H, t, *J* 6.15, H1'), 3.910 (1 H, dd, $J_{4',5'a}$ 3.12, $^2J_{5'a,5'b}$ 11.39, H5'a), 3.824 (1 H, dd, $J_{4',5'b}$ 2.80, $^2J_{5'a,5'b}$ 11.39, H5'b), 3.725 (1 H, m, H4'), 3.616 (1 H, m, H3'), 2.184 (2 H, m, H2'a, H2'b), 1.901 (3 H, d, *J* 1.06, 5-CH_3), 0.915 (9 H, s, $(CH_3)_3CSi$), 0.104 (3 H, s, CH_3Si), 0.099 (3 H, s, CH_3Si).

3'-*O*-Benzyl-2'-*O*-*tert*-butyldimethylsilyl-5'-deoxy-5'-{[*N*-(5'-*O*-*tert*-butyldimethylsilyl-3'-deoxythymidine-3'-yl)carboxamido]methyl}ribosylthymine (VII). Carbonyldiimidazole (0.42 g, 2.6 mmol) was added to a solution of acid (**VI**) (1.1 g, 2.2 mmol) in abs. CH_2Cl_2 (10 ml), and the reaction mixture was stirred for 1 h followed by the addition of amine (**VI**) (0.86 g, 2.4 mmol) in abs CH_2Cl_2 (5 ml). After a night, the mixture was diluted by CH_2Cl_2 (50 ml) and water (50 ml), divided, and the water layer was extracted with CH_2Cl_2 (2 × 20 ml). The extracts were washed with 10% aqueous $KHSO_4$ (2 × 20 ml); saturated aqueous $NaHCO_3$ (2 × 20 ml), and water; dried with anhydrous Na_2SO_4; and evaporated. The residue was chromatographed on a silica-gel column with a methylene chloride–3% ethanol mixture. Dinucleotide (**VII**) was obtained in solid foam form in a yield of 1.5 g (81%), R_f 0.64(B). ^{1}H NMR (here and after, cT is the residue of 5'-deoxy-5'-carboxymethylribosylthymine and Ta is the residue of 3'-amino-3'-deoxythymidine): 11.329 (1 H, bs, H3 cT), 11.288 (1 H, bs, H3 Ta), 8.286 (1 H, d, *J* 7.32, 3'-NH Ta), 7.485 (1 H, bd, *J* 1.16, H6 cT), 7.476 (1 H, bd, *J* 1.09, H6 Ta), 7.26–7.39 (5 H, m, Ph cT), 6.181 (1 H, t, *J* 6.64, H1' Ta), 5.787 (1 H, d, $J_{1',2'}$ 6.06, H1' cT), 4.654 (1 H, d, $^2J_{a,b}$ 11.84, Ha PhC$\underline{H}_2$O cT), 4.582 (1 H, d, $^2J_{a,b}$ 11.84, Hb PhC$\underline{H}_2$O cT), 4.482 (1 H, t, *J* 5.52, H2' cT), 4.297 (1 H, m, H3' Ta), 3.965 (1 H, m, H4' cT), 3.69–3.84 (4 H, m, H3' cT; H4', H5'a,b Ta), 2.218 (2 H, m, H6'a,b cT), 2.117 (2 H, m, H2'a,b Ta), 1.931 (2 H, m, H5'a,b cT), 1.816 (3 H, d, *J* 1.16, 5-CH_3 cT), 1.782 (3 H, d, *J* 1.09, 5-CH_3 Ta), 0.871 (9 H, s, $(CH_3)_3CSi$), 0.814 (9 H, s, $(CH_3)_3CSi$), 0.059 (6 H, s, $(CH_3)_2Si$), 0.038 (3 H, s, CH_3Si), –0.028 (3 H, s, CH_3Si). Mass spectrum: *m/z* 841.6. Calculated 841.14 $[M-H]^-$ ($C_{41}H_{62}N_5O_{10}Si_2$).

3'-*O*-Benzyl-2'-*O*-*tert*-butyldimethylsilyl-5'-deoxy-5'{[*N*-(3'-deoxythymidine-3'-yl)carboxamido]methyl}ribosylthymine (VIII). Water (1 ml) was added to the solution of dinucleotide (**VII**) (1.4 g, 1.7 mmol) in TFA, cooled to 0°C (9 ml), and the mixture was stirred for 20 min at 0°C. The mixture was then poured under vigorous stirring in cooled saturated $NaHCO_3$ (150 ml) and extracted with CH_2Cl_2 (3 × 20 ml). Extracts were washed with saturated aqueous $NaHCO_3$ (2 × 20 ml) and water, dried with anhydrous Na_2SO_4, and evaporated. The residue was chromatographed on a silica-gel column (3.5 × 15 cm) with a methylene chloride–5% ethanol mixture. The yield of compound ((**VIII**) in solid foam form was 1 g, (81%). R_f 0.63 (B). ^{1}H NMR: 11.332 (1 H, bs, H3 cT), 11.242 (1 H, bs, H3 Ta), 8.281 (1 H, d, *J* 7.26, 3'-NH Ta), 7.754 (1 H, bd, *J* 0.85, H6 cT), 7.483 (1 H, bd, *J* 0.65, H6 Ta), 7.26–7.39

(5 H, m, Ph cT), 6.183 (1 H, t, *J* 6.59, H1' Ta), 5.792 (1 H, d, $J_{1',2'}$ 6.16, H1' cT), 5.045 (1 H, t, *J* 5.15, 5'-OH Ta), 4.656 (1 H, d, $^2J_{a,b}$ 11.84, Ha PhC$\underline{H}_2$O cT), 4.588 (1 H, d, $^2J_{a,b}$ 11.84, Hb PhC$\underline{H}_2$O cT), 4.490 (1 H, t, *J* 5.61, H2' cT), 4.321 (1 H, m, H3' Ta), 3.972 (1 H, m, H4' cT), 3.762 (2 H, m, H3' cT; H4' Ta), 3.632 (1 H, m, H5'a Ta), 3.551 (1 H, m, H5'b Ta), 2.13–2.30 (3 H, m, H6'a,b cT; H2'a TA), 2.073 (1 H, m, H2'b Ta), 1.925 (2 H, m, H5'a,b cT), 1.819 (3 H, d, *J* 0.85, 5-CH_3 cT), 1.784 (3 H, d, *J* 0.65, 5 CH_3 Ta), 0.814 (9 H, s, $(CH_3)_3CSi$), 0.039 (3 H, s, CH_3Si), –0.029 (3 H, s, CH_3Si). Mass spectrum: *m/z* 727.4. Calculated 726.88 $[M-H]^-$ ($C_{35}H_{48}N_5O_{10}Si$).

2'-*O*-*tert*-Butyldimethylsilyl-5'-deoxy-5'-{[*N*-(3'-deoxythymidine-3'-yl)carboxamido]methyl}ribosylthymine (IX). Dinucleotide (**VIII**) (0.9 g, 1.2 mmol) in abs. THF (10 ml) was hydrogenated over Pd/C (10%, 0.18 g) at room temperature and atmospheric pressure for 5 h. The reaction mixture was filtered through celite, the residue was washed with THF (4 × 10 ml), and the filtrate was evaporated. Compound (**IX**) was prepared in solid foam form in a yield of 0.76 g (99%), R_f 0.47 (B). ^{1}H NMR: 11.290 (1 H, bs, H3 cT), 11.236 (1 H, bs, H3 Ta), 8.271 (1 H, d, *J* 7.26, 3'-NH Ta), 7.754 (1 H, bd, *J* 0.96, H6 cT), 7.447(1 H, bd, *J* 0.87, H6 Ta), 6.181 (1 H, t, *J* 6.52, H1' Ta), 5.725 (1 H, d, $J_{1',2'}$ 5.11, H1' cT), 5.031 (1 H, t, *J* 4.98, 5'-OH Ta), 4.891 (1 H, d, *J* 5.61, 3'-OH, cT), 4.312 (1 H, m, H3' Ta), 4.207 (1 H, t, *J* 5.05, H2' cT), 3.749 (3 H, m, H3', H4' cT; H4' Ta), 3.629 (1 H, m, H5'a Ta), 3.549 (1 H, m, H5'b Ta), 2.205 (3 H, m, H6'a,b cT; H2'a Ta), 2.075 (1 H, m, H2'b Ta), 1.946 (1 H, m, H5'a cT), 1.836 (1 H, m, H5'b cT), 1.803 (3 H, d, *J* 0.96, 5-CH_3 cT), 1.781 (3 H, d, *J* 0.87, 5 CH_3 Ta), 0.827 (9 H, s, $(CH_3)_3CSi$), 0.039 (3 H, s, CH_3Si), 0.004 (3 H, s, CH_3Si). Mass spectrum: *m/z* 637.5. Calculated 636.75 $[M-H]^-$ ($C_{28}H_{42}N_5O_{10}Si$).

2'-*O*-*tert*-Butylmethylsilyl-5'-deoxy-5'-{[*N*-(5'-*O*-(4,4'-dimethoxytrityl)-3'-deoxythymidine-3'-yl)carboxamido]methyl]ribosylthymine (X). Dinucleotide (**IX**) (0.7 g, 1.1. mmol) was evaporated with abs. pyridine (2 × 5 ml) and dissolved in abs. pyridine (5 ml). $(CH_3)_2TrCl$ (0.44 g, 1.3 mmol) was added and the mixture was stirred for a night. Water (0.5 ml) was then added and stirred for 30 min followed by the addition of triethylamine (0.1 ml). The reaction mixture was evaporated, and the residue was reevaporated with water (3 × 5 ml), dissolved in ethyl acetate (50 ml), washed with saturated $NaHCO_3$ (2 × 20 ml), dried with Na_2SO_4, and evaporated. The residue was chromatographed on a silica-gel column with a methylene chloride–5% ethanol mixture containing 0.2% triethylamine. The yield of compound (**X**) in solid foam was 0.81 g (78%), R_f 0.36 (B). ^{1}H NMR: 11.290 (2 H, bs, H3 cT, H3 Ta), 8.252 (1 H, d, *J* 7.59, 3'-NH Ta), 7.529 (1 H, bd, *J* 0.99, H6 cT), 7.442 (1 H, bd, *J* 1.1, H6 Ta), 6.84–6.99 (13 H, m, DMTr), 6.215 (1 H, t, *J* 6.48, H1' Ta), 5.725 (1 H, d, $J_{1',2'}$ 5.12, H1' cT), 4.892 (1 H, d, 5.98, 3'-OH, cT), 4.476 (1 H, m, H3' Ta), 4.203 (1 H, t, *J* 4.99, H2' cT), 3.876 (1H, m, H4' Ta), 3.734 (2 H, m, H3', H4' cT), 3.734 (6H, s, $(CH_3)_2Tr$), 3.273 (1 H, dd, $J_{4',5'a}$ 4.41, $^2J_{5'a,5'b}$ 10.56, H5'a Ta), 3.171 (1 H, dd, $J_{4',5'a}$ 1.99, $^2J_{5'a,5'b}$ 10.56, H5'b Ta), 2.319 (1 H, m, H2'a Ta), 2.08–2.25 (3 H, m, H6'a,b cT; H2'b Ta), 1.908 (1 H, m, H5'a cT), 1.823 (1 H, m, H5'b cT). 1.792 (3 H, d, *J* 0.99, 5-CH_3 cT), 1.445 (3 H, d, *J* 1.1, 5-CH_3 Ta), 0.824 (9 H, s, $(CH_3)_3CSi$), 0.035 (3 H, s, CH_3Si), 0.001 (3 H s, CH_3Si). Mass spectrum: *m/z* 939.8. Calculated 939.12 $[M-H]^-$ ($C_{49}H_{60}N_5O_{12}Si$).

2'-*O*-*tert*-Butyldimethylsilyl-5'-deoxy-5'-{[*N*-(5'-*O*-(4,4'-dimethoxytrityl)-3'-deoxythymidine-3'-yl)carboxamido]methyl}ribosylthymine-3'-(2-cyanethyl-*N*-diisopropylphosphoramidite) (XI). DIPEA (0.41 g, 0.54 ml, 3.2 mmol) and then 2-cyanethyl-*N,N*'-diisopropylchlorophosphoramidite (0.37 g, 1.58 mmol) were added to the solution of dinucleotide (**X**) (0.74 g, 0.79 mmol) in dry CH_2Cl_2 (5 ml). The mixture was stirred for 2 h, poured into cold saturated $NaHCO_3$, and stirred for 5 min. CH_2Cl_2 (50 ml) was added and the mixture was divided. The water layer was extracted with CH_2Cl_2 (20 ml), organic extracts were washed with saturated $NaHCO_3$ (2 × 20 ml) and saturated NaCl (20 ml), dried with anhydrous Na_2SO_4, and evaporated. The residue was chromatographed on a silica-gel column with a methylene chloride–2% methanol mixture containing 0.2% triethylamine. The yield of compound (**XI**) in solid foam was 0.66 g (74%), R_f 0.46 (A). ^{31}P NMR: 148.464, 146.749. Mass spectrum: *m/z* 1139.8. Calculated 1139.34 $[M-H]^-$ ($C_{58}H_{77}N_7O_{13}PSi$).

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